from the knee joint cavity, in agreement with the previously observed hypocalcaemic effect after i.a. injection.
The efficacy of the HA-sCT treatment was tested in a rabbit OA model, where a chondroprotective effect of the highest dose tested was proven by macro- and microscopic assessments and histological findings. The cartilage surfaces of the weight-bearing parts, such as the medial tibial plateau and the lateral and medial femoral condyle, were evaluated and graded for degradation severity
The average scores on the macroscopic assessment of sCT or HA-sCT treated group indicated a significant reduction in cartilage damage in comparison to controls.
PAS staining of nuclei, indicating the maintenance of chondrocyte number, and Alcian blue staining of proteoglycan, indicating the matrix integrity of the cartilage, were significantly positive in the majority of the HA-sCT treated groups.
Conclusions: The in vivo PK and efficacy/safety profiles of sCT and HA-sCT were examined in this study in view of a potential application in the treatment of OA. Although the i.a. injection of the different sCT-based formulations showed to be comparably chondroprotective in a mechanical model of OA in vivo, the HA-peptide conjugate has the advantage of a sustained action and of a decrease in systemic exposure to sCT with consequent potential safety concerns. The promising results encourage further development and investigation on this therapeutic approach for OA.

869
HYALURONIC ACID ALKYL DERIVATIVE (HYADD®4) SHOWING INHIBITORY ACTIVITY ON HYALURONIDASES

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Purpose: Hyaluronic Acid (HA) homeostasis in the knee is based on a fine balance between synthesis and degradation; in pathological conditions, Hyaluronidases (specifically Hyal-2) play a key role in HA catabolism. The reduction of endogenous HA Molecular Weight (MW) and concentration in Synovial Fluid (SF) is one of the molecular signals of Osteoarthritis (OA) progression. Viscosupplementation is an intra-articular pharmacological therapy based on mechanical restoration of joint performance; to date, no polymer used in viscosupplementation is known to have an inhibitory action on Hyaluronidases. We present here a novel series of HA alkyl-amide derivatives (HYADD®) which, besides good rheological performances, show inhibition of Hyal-2, suggesting a possible biological effect in SF degradation.
Methods: Several unmodified and chemically modified polymers actually used in viscosupplementation therapy were compared for their inhibitory effect on the hyaluronidase enzymatic action towards HA. Therefore, in presence of these polymers, HA (2000 kDa) was incubated at 37°C as a substrate for Bovine Testis Hyaluronidase (BTH) and the resulting MW was determined by means of SEC-TDA Viscotek. In order to avoid the physical entrapment effect, the experiment was repeated after depolymerization of the test materials by heating at 105°C for 24 h, before incubation with HA. Since only the HA hexadecylamide (HYADD®4) was able to preserve the initial HA MW, a series of HA amide derivatives with different alkyl/aryl side chains were tested in terms of Hyaluronidase inhibition, with the aim of verifying the influence of their structure on this activity. C8-, C12-, C15-, C16-, C18- and benzyl- amide of HA were then synthesized and their IC_{50} versus BTH was obtained using HA as substrate and measuring its MW at increasing inhibitor concentration. HYADD®-C16 (HYADD®4) was selected as lead compound on the basis of its good balance between potency and solubility.
Finally, the activity of HYADD®4 (formulated at 8 mg/ml in PBS pH 7) was validated *ex-vivo* in human SFs collected by arthrocentesis from OA patients ($n = 3$); the SFs were not centrifuged in order to avoid the loss of Hyal-2, located on the cell surface. HYADD®4 was incubated at 37°C in the SFs (unmodified HA was used as control); after 24 h $CuCl_2$ 100 mm was added, which allowed the precipitation of proteins and the analysis of the endogenous HA molecular weight.
Results: The MW of HA incubated with BTH in presence of 4 out of 5 polysaccharides tested dropped in just 60 min from 2000 kDa to about 400 kDa; in presence of HYADD®4, even after 24 h, HA preserved its initial MW. The results were confirmed after depolymerization of the polymer: HYADD®4 was still able to completely inhibit BTH, thus preventing HA cleavage.
The comparison of IC_{50} of the different HYADD® derivatives highlighted a strong correlation between the alkyl chain length and the ability to inhibit BTH. Benzyl-, C8- and C-12 derivatives showed no interaction with the enzyme, while C15-, C16- and C18- derivatives proved to be stronger inhibitors (IC_{50} of 276.5 ± 4.7, 166.5 ± 2.6 and 149.7 ± 2.1 μM, respectively). HYADD®4 was therefore selected as lead compound because of its best compromise between solubility and potency.
The *ex-vivo* test on OA SFs demonstrated that HYADD®4 at 425 μM is able to slow down the degradation of endogenous HA by Hyal-2, which is markedly faster in the control test where exogenous unmodified HA was used ($n = 3$, $P < 0.001$).
Conclusions: In this study we found that some alkyl-amide derivatives of HA (HYADD®), compared to other polysaccharides used in the production of viscosupplements, possess the unique ability to inhibit Hyaluronidases. This property was demonstrated in vitro through the measurement of IC_{50} against BTH and then confirmed *ex-vivo* in OA SFs for the selected lead compound, HYADD®4, , which was shown to protect endogenous HA from Hyal-2 degradation. The intra-articular administration of the biopolymer has also the advantage of overcoming easily the possible issues of systemic distribution and chronic toxicity, that are common for the small molecule inhibitors.

870
SELECTIVE INHIBITION OF MATRIX METALLOPROTEASE-13 BY HYALURONIC ACID ALKYLAMIDE DERIVATIVES FOR THE INTRA-ARTICULAR TREATMENT OF OSTEOARTHRITIS

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Purpose: In the pathophysiology of osteoarthritis (OA) several mediators are involved, however the attempts to develop a drug acting on specific targets involved in OA progression (e.g. Matrix Metalloproteases: MMPs) failed at various stages due to unfavourable pharmacokinetics or chronic toxicity. On the other hand Hyaluronic Acid (HA) is widely used as an intra-articular therapy for OA thanks to its capacity of restoring viscoelasticity and lubrication in degraded synovial fluid and to its beneficial effects on inflammation and cartilage metabolism.
We describe here the selection of a HA alkylamide derivative (HYADD®4) with inhibitory activity in the micromolar range *vs* MMP13, for the intra-articular treatment of OA patients.
Methods: *Chlostridium histolyticum* Collagenase (*Ch*C) was used as model of MMP. A number of selected glycosaminoglycans currently used in viscosupplements were compared for their inhibitory effect on *Ch*C enzymatic activity: Chondroitin Sulfate (CS), unmodified HA, HA cross-linked and HA amidated with hexadecylamine, all formulated at 8 mg/ml in PBS pH 7, were incubated in presence of *Ch*C at 37°C and samples withdrawn at different timepoints were tested in a *Ch*C activity assay. In order to abolish the effect of physical entrapment of the enzyme, the test was repeated after depolymerization of the materials by heating at 105°C for 24 h before incubation with *Ch*C.
For the screening of the modified HAs: six amide derivatives of HA were synthesized by alkylation at the carboxylic group of the D-Glucuronic acid unit with a series of linear alkyl chains C8, C12, C15, C16, C18 and a benzyl group, at the same amidation degree. The inhibition constant (K_i) of the depolymerized HA amide derivatives, versus *Ch*C was measured by steady-state kinetics experiments at several inhibitor concentration.
The compound selected as the best *Ch*C inhibitor (HYADD®-C16 or HYADD®4) was then tested in vitro *vs* the catalytical subunits of 10 human MMPs using a MMP Inhibitor Profiling Kit. The selective inhibitory activity of HYADD®4 *vs* MMP13 observed in the screening test was finally validated on the human enzyme in an *ex-vivo* experiment with human inflamed synovial fluid (SF), collected by arthrocentesis and analyzed by means of a human MMP13 assay kit.
Results: In the first screening test the cross-linked HA and HA hexadecylamide, but not CS and high MW HA, showed a relevant decrease of the *Ch*C activity (probe metalloprotease). In order to rule out the hypothesis that the inhibition was due to simple physical entrapment of the enzyme in the test materials, the experiment was repeated after depolymerization of the polysaccharides: in these conditions *Ch*C activity was almost completely recovered in the case of cross-linked HAs, while depolymerized HA hexadecylamide (HYADD®4) caused a further loss of enzyme activity, as a proof of specific inhibition.
Since the HA alkylamide showed this unique property, in the second part of the work six different amide derivatives of HA, synthesized with